Pathophysiology of Stress in Wild and Managed-Care Bottlenose Dolphins (*Tursiops truncatus)*

Patricia A. Fair, Ph.D.
National Ocean Service, Center for Coastal Environmental Health & Biomolecular Research
219 Fort Johnson Road
Charleston, SC 29412
phone: (843) 762-8533 fax: 843-762-8700 email: pat.fair@noaa.gov

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http://www.chbr.noaa.gov

Gregory D. Bossart, V.M.D., Ph.D.
Georgia Aquarium
225 Baker Street NW
Atlanta, GA 30313
phone: (404) 581-4304 email: greg.bossart@gaquarium.org

Award Number: N00014110541
http://www.georgiaaquarium.org

LONG-TERM GOALS

The overall goal of the proposed research is to characterize the pathophysiology of stress in wild and managed-care bottlenose dolphins and to establish relationships between markers of the stress response in cetaceans and immune function, dependent hormonal endpoints, hematology and serum chemistry parameters, biomarkers of stress, inflammation and metabolism and health status.

OBJECTIVES

Objective 1 – To characterize multiple stress markers in managed-care bottlenose dolphins.

Objective 2 – To characterize multiple stress markers in semi-domesticated bottlenose dolphins

Objective 3 – To characterize multiple stress markers in wild bottlenose dolphins

Objective 4 – To integrate the information obtained from these three populations of bottlenose dolphins in order to develop a validated model of stress and its pathophysiologic effects on the bottlenose dolphin.

APPROACH

We plan to assess baseline stress biomarkers in the following three populations of Atlantic bottlenose dolphins (*Tursiops truncatus*): 1) managed-care (Group 1), 2) semi-domesticated (Group 2), and 3)

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wild (Group 3). This approach will provide a truly comparative study among bottlenose dolphins that live under a range of different and varying stressors.

To develop further understanding on stress in wild and managed-care dolphins and the association between classic measures of stress and new technologies, a research team comprised of scientists from federal, academic, and managed-care marine mammal facilities are collaborating on this project to develop integrated measures of stress using a comparative study design. Additionally, this project are partnering with Dr. Dorian Houser and collaborators in a joint effort for the purpose of integrating traditional markers of stress with novel markers of stress.

WORK COMPLETED

SUBTASK 1 - *Collection of samples from Group 1 (managed-care Georgia Aquarium bottlenose dolphins) to characterize multiple stress markers*
A 12 month sample collection period of dolphins from Georgia Aquarium was completed in August 2012. Samples were obtained from 9 individual dolphins with multiple collections during the 12 month period for a total of 32 samples. Several analyses including hematology, immunology and endocrine, have been completed and the remaining analysis will be conducted in FY13. Additionally, 10 dolphins from the navy were sampled on multiple dates with a total of 64 samples collected during the 12-month study as a comparative group.

SUBTASK 2 - *Collection of samples from Group 3 (wild bottlenose dolphins)*
In July 2011 samples were collected from 27 dolphins during capture–release health assessments conducted in the Indian River Lagoon, FL (IRL) as part of the Dolphin Health and Risk Assessment (HERA) Project. All animal capture and sampling protocols for the collection of these samples were conducted under National Marine Fisheries Permit No. 14352 (permit dates from 2009-2014) issued to Dr. Gregory Bossart and approved by the Harbor Branch Oceanographic Institutional Animal Care and Use Committee (IACUC). All samples collected were sent to the appropriate laboratories for analysis and most of these analyses have been completed. Collections of samples from wild dolphins in Charleston, SC have been rescheduled for June 2013.

SUBTASK 3 - *Catecholamine (epinephrine, norepinephrine and dopamine) analysis shall be determined on samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins). These analyses will be conducted by Dr Tracy Romano.*
Catecholamine analyses were completed on the first several months of collections from Group 1 and Group 2 dolphins. All remaining samples from Group 1 and 2 have been sent to Dr. Romano and these are due to be analyzed early in FY13. Catecholamine analysis has been completed on the Group 3 IRL dolphins.

SUBTASK 4 - *Immunological assessments for immunophenotyping (B+T cell lymphocyte subsets, MHCII expression) shall be determined on samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins). These analyses will be conducted by Dr Tracy Romano.*
All immunophenotyping assessments have been completed on Group 1, Group 2 and the Group 3 IRL dolphins.

SUBTASK 5 - *Immunological assessments for* lymphocyte proliferation, natural killer cell activity *shall be determined on samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins). These analyses will be conducted jointly by Drs. Peden-Adams and Fair.*
All immunological assessments for this task (i.e., lymphocyte proliferation and natural killer cell activity) have been completed on Group 1, Group 2 and the Group 3 IRL dolphins.

SUBTASK 6 - *Immunological analysis of IgG, CRP, and pathogen ELISAs shall be determined on samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins). These analyses will be conducted by Dr. Charles Rice. Samples for these analyses will be collected from dolphins as described in Table 2.*
All immunological assessments for this task have been completed on Group 1, Group 2 and the Group 3 IRL dolphins.

SUBTASK 7 - *The following cytokines shall be determined (IL4, IL10, IL17, CD69, TNFα, IFNg, IFNa, MX1, IL2-Ra, FADD) in the collected blood samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins) as outlined in Table 2. These analyses will be conducted by Dr. Jeff Scott.*
All immunological cytokine assessments have been completed on Group 1, Group 2, and the Group 3 IRL dolphins.

SUBTASK 8 – *Proteomic analysis of samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins) will be conducted by Dr. David Janz.*
This work involves using an antibody-based protein microarray, initially developed for grizzly and brown bears, to determine expression levels of 33 stress-associated proteins in small biopsy samples collected from bottlenose dolphins. The microarray measures expression of proteins associated with four key aspects of the stress response: hypothalamic-pituitary-adrenal (HPA) axis, apoptosis/cell cycle, proteotoxicity, and oxidative stress/inflammation. The primary goals in year 1 of this project with dolphins were to (1) evaluate the applicability of the microarray to dolphin skin biopsy specimens, (2) determine whether the microarray could reliably measure stress proteins in white blood cells (WBCs) collected during routine blood sampling of dolphins, and (3) if successful, begin to determine stress protein expression levels in skin and WBC samples. We predicted that many of the 33 antibodies on the microarray would specifically recognize dolphin proteins since they were initially selected from a large panel (>250) of broadly reactive commercially available antibodies.

We have made significant and exciting progress to date with 8 skin samples from wild dolphins received in March 2012. Similar to the approach used with cDNA (gene expression) microarrays, protein expression of individual skin samples (labeled with the fluorescent dye, Cy5) was compared to a pooled standard comprised of equal amounts of each sample (labeled with a different dye, Cy3). The results show that all 33 antibodies on the microarray specifically recognize dolphin proteins, a major finding illustrating its applicability to this species. Among the limited number of skin samples analyzed to date, 7 proteins showed a >5-fold difference among dolphins, with an additional 12 proteins showing a >2-fold difference among dolphins. These results suggest biologically significant differences in stress-associated protein expression among dolphins that may be linked to pathophysiological status. When additional skin samples are analyzed, these proteomic results may be extremely useful when interpreting and integrating results obtained from other investigators on this

project, which will range from the gene expression level through to the whole animal (physiological) level.

Also, we also generated exciting preliminary results using WBCs, which we have never attempted. Alongside the 8 skin samples we ran a single sample of WBCs on the microarray. The results from this sample indicated that 25 of the 33 antibodies recognize proteins present in WBCs. Future work with more concentrated WBC samples may increase this number of proteins, as protein detection may have been limited by the relatively low protein quantity run on the microarray compared to skin.

All samples as indicated in SubTasks 2 and 3 were sent by NOAA/NOS to Dr. Janz under CITES Permit 12US79851A/9 in August 2012 for proteomic analysis. Additional skin and WBC samples will be completed in year 2. In addition, plasma samples were also collected that may be able to be concentrated sufficiently in order to generate data from the microarray. However, although several proteins detected by the microarray are known to be present in blood plasma (e.g., hormones, certain heat shock proteins), many of the proteins are intracellular (e.g., cell signaling molecules, receptors, enzymes) and are unlikely to be detected.

SUBTASK 9 - *Metabolomic analysis of samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins) will be conducted by Dr. Al Dove in association with colleagues at Georgia Tech, Atlanta, GA. Samples for these analyses will be collected from dolphins as described in Table 2.*
All samples for metabolomic analysis were sent to Dr. Dove from collections thus far as indicated in Subtasks 2 and 3. The GC-MS analytical protocols have been refined and samples from 2011 wild IRL dolphins are currently being analysed in the laboratory of Dr. Styckzynski at Georgia Tech. Initial results are promising: multivariate analysis by Principal Components Analysis shows metabolic differentiation between "healthy" and "diseased" animals, although not between "healthy" and "concerned". These analyses will continue, after which discriminant functions will be generated that determine which analytes drive the differentiation of healthy and diseased animals. Once stress measurements from this study are available these will be integrated with metabolic profiles to identify key metabolic markers. The discriminant analyses may indicate which analytes may be most useful as metabolic biomarker candidates in dolphins, as well as revealing underlying metabolic mechanisms of diseases. Samples have been received from Group 1 Georgia Aquarium dolphins and the Group 2 Navy animals and analyses of these will follow completion of the Group 3 IRL samples.

SUBTASK 10 - *Data Management, Quality Assurance and Analysis*
The Microsoft Access database framework was completed to include all variables for the data collected in this study. Thus far, data has been entered for all parameters which have been completed and submitted from the various researchers and laboratories using Quality Assurance/Quality Control processes.

RESULTS

The project was initiated in June 2011 as funding was received by both Dr. Fair (N0001411IP20081) and Bossart (N000141110541). Below are listed several accomplishments and further information is presented under subtasks for FY12.

1. A 12-month sample collection period from Group 1 managed-care dolphins at Georgia Aquarium (n=32 samples) was completed in August 2012.

2. A 12-month sample collection period was completed in August 2012 in partnership the Marine Mammal Program on a collaborative study with Group 2 semi-domesticated dolphins (n= 56 samples) at the U.S. Navy.
3. All samples from the 12-month collections of Group 1 and Group 2 dolphins were shipped to investigators within 2 weeks of the completed study. A CITES permit application was submitted and approved and all collected samples were shipped to Dr. Janz in Canada for proteomic analysis.
4. Sample analyses have been completed for hematology and immunology tests for all samples collected thus far. Completion of the remaining tests and measurements are in process.
5. Data on hematology, serum chemistry, immune, hormone and stress biomarker data that have been completed for this project from the various researchers and laboratories have been entered into the relational database developed for this project. Based on the samples collected thus far and the number of tests being done there will be over 14,000 data points for this study.
6. Presentation: Houser, D.S., Champagne, C., Bossart, G.D., Fair, P.A. 2011. Estimating the impact of specific stressors requires comparisons to minimal stress conditions. Presentation at the Stress Workshop, Marine Mammal Biennial Conference, Tampa, FL., December 2011.

This project is on target for meeting the outlined objectives within specified timeframe.

IMPACT/APPLICATIONS

Well-characterized baseline stress evaluation using classic stress hormones paired with biomarker expression using new technologies will provide needed information on natural variation and inter-relationships in hormones/biomarkers among different matrices and across populations maintained under differing environmental conditions. The assessment of stress variables and response in managed-care animals will have important implications for the assessment and interpretation of stress in wild bottlenose dolphins. Approaches and results developed in this proposal to assess the measurement and burden of stress may also be generalized to other marine mammal species.

In order for the US Navy to understand and assess the physiological condition of animals in the wild, particularly in regions where animals are exposed to acoustic and other anthropogenic stressors, it is important to determine the relationship of stress measures not only between tissue matrices but also between managed-care and wild dolphins. This proposal addresses this critical need and furthermore incorporates the use of new technologies to provide an integrative measure of stress with classic parameters extending our knowledge and application of such measures.

RELATED PROJECTS

The Dolphin Health and Risk Assessment Project has several in-press or submitted publications that are related or applicable to studies on stress including:

Mazzaro, L.M., Johnson, S.P., Fair, P.A., Bossart, G., Carlin, K.P., Jensen, E.D., Smith, C.R., Andrews, G.A., Chavey, P.S., Venn-Watson, S. 2012. Iron Indices among bottlenose dolphins (*Tursiops truncatus*): Identifying populations at risk for iron overload. Comparative Medicine *(in press)*.

Goldstein, D., Schaefer, A.M., Reif, J.S. McCulloch, S.D., Fair, P.A., Bossart, G.D. Clinicopathologic findings from Atlantic bottlenose dolphins (*Tursiops truncatus*) exhibiting cytologic evidence of gastric inflammation. Journal of Zoo and Wildlife Medicine *(in press)*.

Cray, C., Arheart K., Leppert, L., Roberts, K., McCulloch, S., Goldstein, J., Gonzalez, C., Fair, P.A., Bossart, G. Acute Phase Protein Quantitation in Serum Samples from Healthy Atlantic Bottlenose Dolphins (*Tursiops truncatus*) *(submitted)*.

G. Bossart, K. Arheart, L. Leppert, K. Roberts, S. McCulloch, J. Goldstein, C. Gonzalez, J. Sweeney, R. Stone, P.A. Fair, C. Cray. Protein Electrophoresis of Serum from Healthy Atlantic Bottlenose Dolphins (*Tursiops truncatus*) *(submitted)*.